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Construction of a fully active truncated alternansucrase partially deleted of its carboxy-terminal domain

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Abstract **Recombinant expression of the large alternansucrase (2057 amino acids) was hindered in *E. coli* due to poor enzyme solubility and protein degradation. The effects of deletions of the alternansucrase C-terminal CW-like and APY repeated motifs on enzyme solubility and specificity were investigated. A truncated variant deleted of the APY repeats but harboring four C-terminal CW-like repeats displayed a high specific activity and the same specificity of product synthesis as the native enzyme. It is more soluble and suffers less degradation than full length alternansucrase. Hence this truncated variant is a promising tool for the further structural and kinetic study of this interesting enzyme.**

Keywords: Alternansucrase; Glucansucrase; Domain truncation; Amino acid repeats; Cell wall binding repeats

1. Introduction

Alternansucrase (ASR) (EC 2.4.1.140) from *Leuconostoc mesenteroides* NRRL B-1355 is an uncommon glucansucrase that synthesizes from sucrose a high molecular weight polymer (alternan) containing 56% of α-1,6 and 44% of α-1,3 linked glucosyl residues mainly alternately linked [1–3]. ASR retains this alternating specificity even when using maltose as the acceptor during the oligosaccharide synthesis [3,4].

Production and purification of alternansucrase from the native strain is a challenging task, especially as two other contaminating glucansucrases are produced [5]. Moreover, the enzyme production is induced by sucrose thus the partially purified alternansucrase is often contaminated by the co-synthesized polymers. Some strategies to obtain constitutive or alternansucrase enriched mutants were described [6–8] but none of them solved the problems of high molecular weight polymer and glucansucrases contaminations. Heterologous protein production in *Escherichia coli* was also attempted [9,10], however, the expression level was low (160 U l^{-1} of culture [9]) compared to 1730 U l^{-1} obtained with the native strain [3] and no information about the quality of the protein produced was reported.

Based on primary structure ASR has been classified in the glycoside-hydrolase (GH) family 70 [11]. The *asr* sequence revealed that the ASR enzyme at 229 kDa is much larger than the other glucansucrases but shares the same organization in three domains [9] as predicted by amino acid alignments since at present there is no three-dimensional structural data available for the family 70. ASR has an N-terminal domain composed of a long variable region (300 amino acids compared to 130 for other glucansucrases) followed by a highly conserved catalytic domain (974 amino acids like other glucansucrases) that contains the residues which are proposed to be involved in the double displacement mechanism of α-retaining transglycosidases [12]. Finally, ASR ends with a C-terminal domain (709 amino acids compared to 500 for other glucansucrases) commonly named glucan binding domain (GBD) in glucansucrases. However, Shah et al. did not detect any binding to dextran or alternan with alternansucrase compared to streptococcal glucansucrase GTF-I [13]. Accordingly, designation of GBD should be revised for alternansucrase enzyme.

The C-terminal domain is composed of two different series of homologous repeating units in alternansucrase, the CW-like repeats and the APY repeats. The CW-like repeats are homologous to the cell wall binding family motif PFO1473 of the Pfam database [14]. They are 20 amino acid long motifs with a high representation of conserved glycine and aromatic residues. In addition to cell wall binding function, these repeats were shown to be responsible for glucan binding in some glucansucrases [13]. In the ASR sequence, some of CW-like repeats are also found in the N-terminal variable region (Fig. 1A). More striking is the presence of a series of novel repeats named APY repeats that have not been previously found in glucansucrases [15]. Besides, this motif is only found in the inulosucrase from *L. citreum* were it is proposed to improved the enzyme thermal stability [16]. An APY repeat is a 79 residue long motif, again with a high number of conserved glycine and aromatic residues, but is specifically characterized by the presence of the three consecutive residues alanine, proline and tyrosine. In ASR, seven APY repeats are found within the final 550 residues of the C-terminal domain (Fig. 1B).

In this paper, we investigated the heterologous overexpression of alternansucrase gene with the aim of producing a fully active truncated form in *E. coli* in order to get better

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Abbreviations: GBD, glucan binding domain; ASR, alternansucrase; CW, cell wall binding repeats; HP-SEC, high pressure size exclusion chromatography; GH, glycoside-hydrolase

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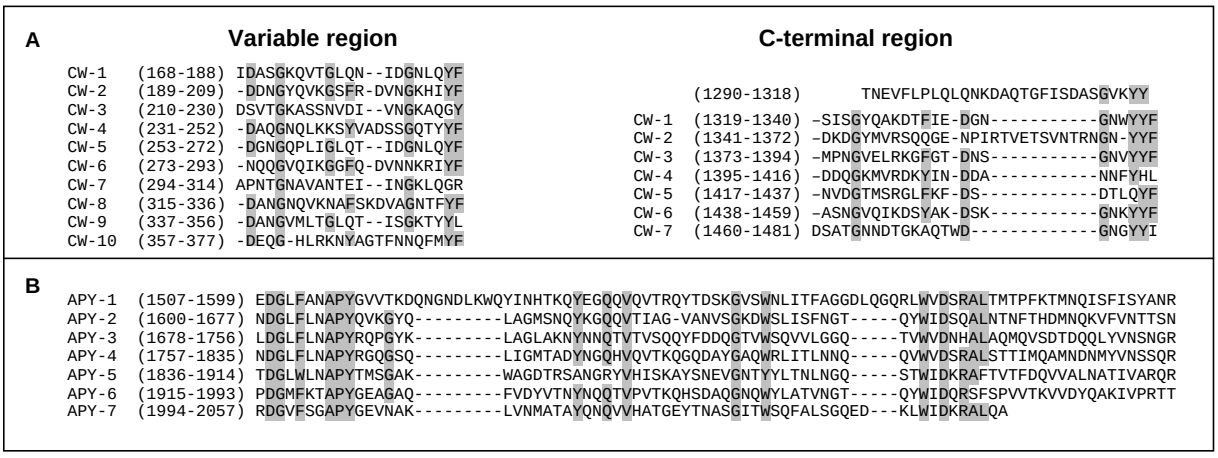

Fig. 1. Alignment of the putative CW-like and APY motifs of ASR. (A) CW-like repeats found in the N-terminal and C-terminal region, in grey: conserved residues defining the motif Pfam PF01473. (B) APY repeats of the C-terminal domain, in grey conserved residues defining the motif.

expression levels and less protein degradation. We also examined, through the characterization of truncated variants, the possible role of CW-like and APY repeats located at the C-terminal end of the protein with respect to alternansucrase activity and specificity.

2. Materials and methods

2.1. Production of native alternansucrase

Native alternansucrase was obtained from culture of *L. mesenteroides* NRRL B-23192 (NCAUR stock culture collection in Peoria, IL), a mutant strain of NRRL B-1355 enriched in alternansucrase [7], grown on standard medium as previously described [17]. Cells and other insolubles were recovered by centrifugation and used as the native alternansucrase source.

2.2. Production of recombinant alternansucrase and truncated variants

E. coli One Shot TOP10 (Invitrogen) was used to transform the constructed plasmids and to express the truncated *asr* genes. Bacterial cells were grown on LB medium with 100 μg ml^{-1} of ampicillin. The induction was performed using 0.02% arabinose (w/v) at $OD_{600\,nm}$ of 0.6. After 15 h following the induction ($OD_{600\,nm}$ about 2.5 or 3.9 g l^{-1} of biomass) cells were harvested by centrifugation (4500 × *g*, 10 min, 4 °C) and resuspended in lysis buffer (20 mM sodium acetate buffer, pH 5.4; Triton X-100, 1%; lysozyme, 1 mg ml^{-1}; DNaseI, 5 mg ml^{-1}) to an optical density $OD_{600\,nm}$ of 80. Cells were disrupted by sonication. The protein extracts obtained were centrifuged (27000 × *g*, 30 min, 4 °C). The supernatants were tested for protein content resulting in concentration of 12 ± 1 g l^{-1} and were tested for activity. The amount of recovered activity was named soluble activity and expressed in U l^{-1} of culture. The pellet of sonication was resuspended and tested for activity. It was named insoluble activity and was also expressed in U l^{-1} of culture. Renaturation of the pellet was also tested by 1 h incubation in urea 8 M, pH 8.0, followed by overnight dialysis against sodium acetate buffer 20 mM, pH 5.4.

2.3. Cloning of the asr truncated genes

Restriction enzymes were purchased from New England Biolabs and used according to the manufacturer's instructions. DNA purification was performed using QIAquick (PCR purification and gel extraction) and QIAprep (plasmid purification) from Qiagen. Genomic DNA of *L. mesenteroides* NRRL B-1355 strain was extracted with Blood and Cell Culture DNA Kit from Qiagen. DNA sequencing was carried out by Genome Express (Grenoble, France). PCR reactions were set up using the Expand Long Template PCR System (Roche Diagnosis Corporation).

The alternansucrase gene (GenBank accession no. AJ250173) was amplified by PCR using genomic DNA as a template with the following primers: Bad dir (forward) 5′-gccatggaacaacaagaaacagttacccgt-3′; Bad inv (reverse) 5′-agcttgcaaagcacgcttatcaatccatagc-3′; Bad var del (forward) 5′-gccatggtaacagggttgcaaactatttcagga- 3′; Bad C-del (reverse) 5′-cgtttttgcaatgttaaaatactggttagtagccca-3′; Bad C-del 2 (reverse) 5′-ccctcgagacatagtcccatcaacatttaagtg-3′. The amplified *asr* gene fragments were cloned into the pBad/Thio-TOPO vector (Invitrogen). The in-frame cloning enabled upstream fusion with the thioredoxin gene and downstream fusion with the 6×His tag gene. The forward primers contain a *Nco*I restriction site (ccatgg) including a start codon designed to remove the thioredoxin. The clones were sequenced to confirm that no misincorporation had occurred. The [pBad *asr entier*], [pBad *asr C-APY-del*], [pBad *asr C-del*] and [pBad *asr core*] plasmids were, respectively, constructed with the following primer couples: Bad dir/Bad inv, Bad dir/Bad C-del 2, Bad dir/Bad C-del and Bad var del/Bad C-del. The [pBad/Thio] plasmid provided by the supplier was used as expression control.

2.4. ASR C-APY-del purification

The thioredoxin gene was removed from the [pBad *asr C-APY-del*] plasmid to give the [pBad *asr C-APY-del* Δ*thio*] that was used for ASR C-APY-del production. The affinity purification was performed with ProBond nickel-charged resin (Invitrogen) following the manufacturer's instructions. ASR C-APY-del was eluted with 250 mM imidazole and then dialyzed against the activity buffer. The protein concentration was determined by the Bradford method [18] using the Bio-Rad reagent (Bio-Rad Laboratories) and bovine serum albumin as standard.

2.5. Activity assay and acceptor reaction

Activities were determined using non purified fractions of *E. coli* extract as no other glucansucrase or sucrose acting enzyme is produced by the strain. One glucansucrase unit is defined as the enzyme quantity that catalyses the release of 1 μmol of fructose per minute at 30 °C in a sodium acetate buffer 20 mM, pH 5.4 and 100 g l^{-1} of sucrose. Released reducing sugars were quantified by the DNS method [19] using fructose as reference. Released glucose were checked by HPLC to be less than 5% which confirmed that constructed variants still possesses transglucosidase activity and not simply hydrolase activity.

The acceptor reaction was performed with 100 g l^{-1} of sucrose and 50 g l^{-1} of maltose in the same conditions as the activity assay. Completion of sucrose consumption and oligosaccharides produced were monitored by HPLC on a C18 column (5 μm, 250 × 4 mm, Bishoff chromatography) with deionised water at a flow rate of 0.5 ml min^{-1}.

2.6. SDS–PAGE and zymogram

SDS–PAGE analyses were performed using the Nu PAGE Novex 3–8% Tris–acetate gels (Invitrogen). Gels were stained with Colloidal Blue Staining Kit (Invitrogen). The Prestained Precision Protein Stan-

dard was purchased from Bio-Rad Laboratories. Colloidal Blue stained gels were loaded with 15 µl samples of *E. coli* extract of identical cell density ($OD_{600\,nm}$ of 80, see Section 2.2). Zymograms to detect activity were performed by in-gel protein renaturation with the activity buffer, incubation in 100 g l^{-1} sucrose and detection of the synthesized polymer with Schiff's reagent (Sigma-Aldrich) [20]. About 0.1 U was loaded per well.

2.7. Glucan structure analysis

Glucans were produced from sucrose using 0.5 U ml^{-1} of soluble enzyme in the conditions of the activity assay. Sucrose depletion was monitored by HPLC using a C18 column. The reaction was stopped by 5 min incubation at 95 °C. The mixture was then centrifuged for 5 min at 5000 × *g* to remove precipitated proteins and analyzed by high pressure size exclusion chromatography (HP-SEC) on a Shodex SB-805 HQ and a SB-8025 HQ gel permeation chromatography column connected in series at 50 °C with a flow rate of 1 ml min^{-1} of NaCl 50 mM. The calibration standards used were dextrans of 2000, 500, 70, and 10 kDa, isomaltotriose, sucrose and fructose. The high molecular weight polymer was precipitated by addition of 1 vol. of ethanol, recovered by centrifugation, washed three times with water and freeze-dried before ^{13}C NMR analysis.

The ^{13}C NMR (75.468 MHz) spectra were recorded on a Bruker Avance 300 spectrometer. Glucans were dissolved at 50 mg ml^{-1} in D_2O. Carbon spectra were recorded at 333 K, 1.445 s acquisition time and 12288 scans were accumulated.

Polymer acetolysis was performed as described elsewhere [21]. The reaction products were analyzed by capillary electrophoresis in electroosmotic mode with a borate buffer in conditions enabling the separation of disaccharide regioisomers as described by Joucla et al. [22].

3. Results and discussion

3.1. Design of ASR truncated forms

Cloning of the full length ASR encoding gene was first performed in fusion with the thioredoxin and 6×His encoding genes at the 5′ and 3′ ends, respectively. To investigate the functions of the CW-like and APY repeats in respect to alternansucrase activity and specificity, three truncated forms were constructed (Fig. 2). The construct ASR C-APY-del has all of the APY repeats removed but retains just 4 of the CW-like repeats at the C-terminal. The construct ASR C-del has the whole C-terminal domain including the CW-like and APY repeats removed. Finally, the construct ASR core corresponds to the catalytic domain alone. It was designed with reference to a conceptually similar truncated form mutant of GTF-I (the mutansucrase from *Streptococcus downei)* which was described as retaining the wild type activity of the full enzyme [23].

3.2. Overexpression of ASR and truncated forms

The wild type and truncated constructs were successfully overexpressed in *E. coli* as attested by the colloidal blue stained SDS–PAGE. However, the over-produced proteins were mainly recovered in the insoluble extracts (the pellet harvested after cell sonication), the highest amount of soluble protein (recovered in the post-sonication supernatant) being obtained with the ASR C-APY-del construct (Fig. 3A, lane 2). Both soluble and insoluble ASR extracts were tested for transglucosidase activity. The full length alternansucrase was produced at a level of 661 U l^{-1} of culture (Fig. 2), which is a 4-fold increase compared to previous work [9] but only 26% of the total activity was recovered in the soluble extract. ASR core and ASR C-del activity recovered in soluble extract was very low and accounted for less than 1% of the ASR activity, most of the protein being insoluble and also inactive. The removal of the entire C-terminal domain was not favourable for protein expression. On the contrary, with 774 U l^{-1}, ASR C-APY-del was produced with activity level similar to wild type. Most

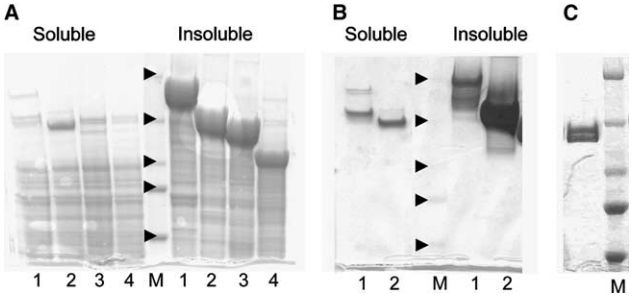

Fig. 3. SDS–PAGE of the produced alternansucrase and truncated variants. (A) Colloidal Blue stained gel. (B) Zymogram detecting the polymer produced in situ. (C) Colloidal Blue stained gel of purified ASR C-APY-del deleted of the thioredoxin. Loaded samples are ASR (lane 1), ASR C-APY-del (lane 2), ASR C-del (lane 3) and ASR core (lane 4). Soluble: sonicated extract supernatant. Insoluble: sonicated extract pellet. M, molecular weight marker of 250, 150, 100, 75 and 50 kDa. Arrows indicate the molecular weight markers.

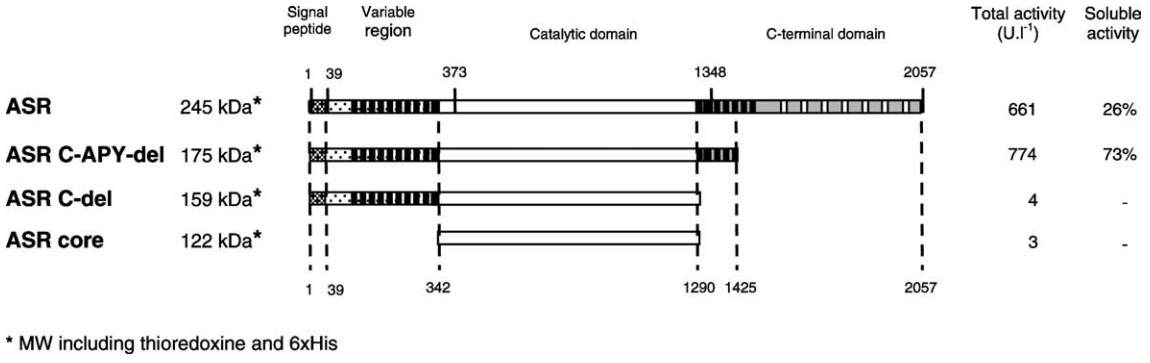

Fig. 2. Truncated ASR constructions. Upper numbering corresponds to amino acids delimiting domains of alternansucrase as defined by Arguello-Morales et al. [9]. Lower numbering corresponds to amino acids delimiting the designed truncations. Black stripes ■■■ represent CW-like repeats. White stripes □□□ represent APY repeats. Total activity is the activity produced in U l^{-1} of culture, recovered after sonication. Soluble activity: percentage of total activity recovered in the sonicated extract supernatant.

of the activity recovered during expression of this construct was soluble and accounted for 73% of the total activity produced (Fig. 2). Zymograms of ASR extracts showed that ASR suffered from a significant level of protein degradation. The major active species in the insoluble extract is observed at 245 kDa corresponding to the full length ASR. In the soluble fraction, ASR was again very much degraded, the major band observed corresponding to a 168 kDa degraded form (Fig. 3B). The use of antibodies against thioredoxin or 6×His tag on Western blot revealed that the degradation occurred from both protein extremities (data not shown). The expression by BL21 *E. coli* strains deficient in the La and OmpT proteases did not reduce the degradation. In contrast, ASR C-APY-del was also significantly less degraded than the entire ASR as revealed in Fig. 3B, lane 2. A renaturation of the proteins harvested in the ASR C-APY-del insoluble extracts yielded the recovery of 3000 U l^{-1} of culture, showing that insolubility was mainly due to the formation of potentially active inclusion bodies.

3.3. Contribution of the APY motif to ASR specificity

The APY repeats are found in alternansucrase but in no other glucansucrases of GH family 70 and could thus be proposed to play a role in the unique ability of this enzyme to synthesize alternating linkages. To examine this assumption, the products formed by ASR C-APY-del were compared to those synthesized by the native alternansucrase from *L. mesenteroides* NRRL B-23192 [7]. First, size exclusion chromatography (HP-SEC) chromatograms of the products obtained from 100 g l^{-1} sucrose showed that both enzymes synthesize a polymer with an estimated molecular weight of 1700 kDa corresponding to about 10000 glucosyl units (peak 1, Fig. 4). Both chromatograms also revealed the presence of oligosaccharides never described before with an estimated molecular weight of 1.3 kDa (peak 2, Fig. 4). We did not observed this peak at initial time or in control reaction involving extract without alternansucrase. The presence of these oligosaccharides was also confirmed by high pressure anionic exchange chromatography, detecting many oligosaccharides displaying overall an average degree of polymerization of 8. These oligosaccharides are 10-fold more abundant in the ASR C-APY-del reaction medium. Nevertheless, the deletion of APY repeats did not suppress polymerase activity showing that this motif is not involved in polymer elongation.

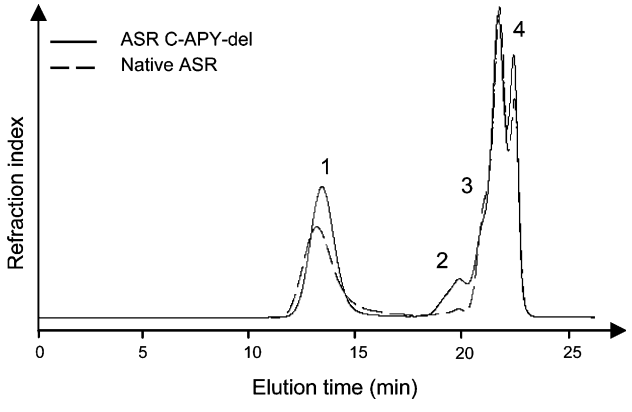

Fig. 4. High performance size exclusion chromatography analysis of products synthesized by the native alternansucrase from *L. mesenteroides* NRRL B-23192 and ASR C-APY-del. (1) Polymer of 1700 kDa, (2) oligosaccharides of 1.3 kDa, (3) disaccharides, and (4) monosaccharides.

To investigate whether the APY motifs were involved in the ability to form α-1,6 and α-1,3 alternated linkages, we analyzed the structure of the high molecular weight glucans synthesized by alternansucrase and ASR C-APY-del. ^{13}C NMR spectra were identical for both polymers and indicated the occurrence of α-1,6 and of α-1,3 linkages (Fig. 5). These spectra are very similar to the spectrum of alternan synthesized by the native strain *L. mesenteroides* NRRL B-1355 described by Seymour et al. [1]. The integration of the signals corresponding to the anomeric carbons on ^{13}C NMR spectra indicated that 44% of the glucosyl residues were α-1,3 linked and 56% were α-1,6 linked and this was corroborated with the integration of anomeric proton signals of ^{1}H NMR corresponding to 42% and 58%, respectively. Quantification of the glucosyl moieties was also attempted by GC–MS after methylation and hydrolysis (data not shown). The results obtained are similar between

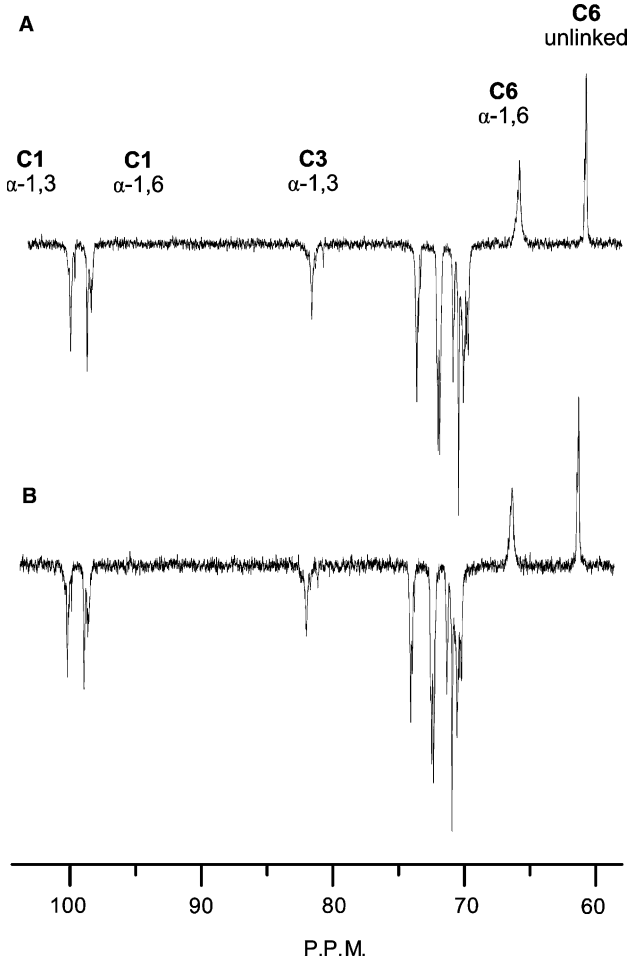

Fig. 5. ^{13}C NMR spectra of the polymers synthesized by: (A) native ASR from *L. mesenteroides* NRRL B-23192 and (B) ASR C-APY-del. Peaks pointing upwards correspond to carbons carrying even number of protons (C6) and peaks pointing downwards correspond to carbons carrying odd number of protons (other carbons). The anomeric region presents two carbons of similar intensity corresponding to the α-1,6 and α-1,3 at 100.2 and 98.5 ppm, respectively. The C6 and the C3 engaged in the glucosidic linkages were identified at 66.4 and 82.5 ppm, respectively and the C6 not engaged in the linkage is located at 61.2 ppm [1].

polymers and are analogous to the structural analysis performed on alternan produced by *L. mesenteroides* NRRL B-1355 [1] or by the heterologous expressed recombinant alternansucrase [10]. Structural data of the polymer produced by ASR C-APY-del are consistent with the structure proposed by Seymour et al., composed of alternated α-1,6 and α-1,3 linkages in the backbone-chain and branches of consecutive α-1,6 linked glucosyl moieties accounting for the excess of α-1,6 linked glucosyl residues compared to those of α-1,3. To check that the α-1,6 and α-1,3 linkages occur in an alternating pattern and not at random, an acetolysis of the polymers was carried out. As the α-1,6 linkages are 30 times more sensitive to acetolysis compared to the α-1,3 linkage [21], the reaction should essentially release glucose and nigerose (Glcp-(α1 → 3)-Glc) in the case of an alternated motif. The products of the reaction were analyzed by capillary electrophoresis in conditions that enabled the disaccharide regioisomers to be separated [22]. As expected, the principal degradation products were glucose and nigerose. Presence of nigerose, 25% and 16% for ASR and ASR C-APY-del polymers, respectively, provides evidence of the occurrence of α-1,6 and α-1,3 alternating motifs. According to the structural analysis, the polymer synthesized by the ASR C-APY-del is identical to the one obtained by native alternansucrase. It can be concluded that, contrary to our suspicions, the APY motifs are not involved in the specific formation of alternating α-1,6 and α-1,3 linkages. In addition, no decrease in thermal stability was observed for the truncated enzyme suggesting no similar role to that of the inulosucrase from *L. citreum* [16]. A construct of *asr C-APY-del* without thioredoxin tag was then expressed into *E. coli* and the soluble extracts purified by affinity chromatography on nickel column. The purified fraction analyzed by SDS–PAGE revealed that two proteins of 147 and 134 kDa were obtained (Fig. 3C) and the N-terminal amino acid sequencing of the two forms showed that one form corresponds to the maturated ASR C-APY-del devoid of the 39-residue signal sequence and the second one lacks the next 102 residues, hence starting at amino acid 142. Both bands, cut from electrophoresis gels, catalyzed the formation of oligoalternans by acceptor reaction [3]. The specific activity of this purified preparation was of 160 U mg^{-1}. It corresponds to a turnover of 404 s^{-1} in the assay conditions, ranking ASR C-APY-del among the most efficient enzyme glucansucrases.

Our results show that expression of alternansucrase encoding genes and truncated forms by *E. coli* results in the production of insoluble proteins forming inclusion bodies. Contrary to what was suspected, the APY repeats found in the C-terminal domain of the alternansucrase are neither involved in polymerase activity nor in specificity but influence the protein solubility. Indeed when they are removed, the solubility is clearly improved. From this observation, we can suggest that the APY repeats, rich in aromatic residues, strongly influence protein aggregation via protein–protein hydrophobic interactions. Furthermore, APY repeats of the C-terminal end influence the susceptibility of ASR toward protein degradation, less degraded forms are produced in their absence. A possible explanation could be due to the presence of a high percentage of rare codons in this region that could impede the translation process thus exposing the partially folded protein to protease attack.

In conclusion, ASR C-APY-del is more soluble and less degraded than the full length ASR. In addition, it displays a high turnover and the same product specificity as that of ASR. Hence this truncated variant is a promising tool for the further structural and kinetic study of alternansucrase.

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